J. HYM. RES.
Vol. 3, 1994, pp. 151-156

Localization of the Female Sex Pheromone Gland in *Cotesia rubecula* Marshall (Hymenoptera: Braconidae)

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Abstract.- A combination of behavioural, histological and electron microscopic techniques was used to localize the female sex pheromone gland in the parasitic wasp *Cotesia rubecula* (Marshall) (Hymenoptera: Braconidae). The genital capsule was identified as the source of the sex pheromone by presenting males with a choice of body parts in a flight tunnel. Histological examination and electron microscopy further reduced the possibilities for pheromone production to two structures. One of these was a gland in a position similar to that reported for other braconid wasps, and which was associated with surface pores but lacked any obvious secretory products. The other was an active secretory gland which is associated with the moving parts of the ovipositor. Further studies aimed at isolating and identifying the chemicals involved are required to resolve this issue.

The presence of sex pheromone glands has been demonstrated in a number of species of parasitic wasps (Weseloh 1976, 1980; Tagawa 1977, 1983). Although unable to localize the sex pheromone source more precisely than the abdominal region, Vinson (1978) suggested that Dufour's gland produced a sex identification pheromone in *Cardiochiles nigriceps*. Obara and Kitano (1974) first demonstrated that the source of the female sex pheromone in *Cotesia glomerata* is the tip of the abdomen, near the ovipositor. Tagawa (1977) later described a pair of secretory glands located at the base of the second valvifer on the ninth abdominal tergite of this species, which he suggested was responsible for sex pheromone production. A subsequent study (Tagawa 1983) revealed the existence of similar glands in a corresponding position in all seven braconid species examined. Upon revising his initial findings (Weseloh 1976), Weseloh (1980) likewise concluded that paired epidermal glands on the last abdominal tergite were the sex pheromone source in *Cotesia melanoscelus*.

Previous field observations (Keller unpublished) established that courtship in male *Cotesia rubecula* (Marshall) (Hymenoptera: Braconidae) is elicited by a female sex pheromone. The present study combined behavioural tests with microscopic techniques in order to identify the position of the sex pheromone gland in *C. rubecula*.

MATERIALS AND METHODS

Behavioural Experiments.—*C. rubecula* was reared in the laboratory on larvae of *Pieris rapae* L. using the methods of Keller (1990). Females between one and four days of age were frozen at -15° C for approximately 30 minutes before being dissected.

Using the same experimental protocol, we performed two experiments to test the responsiveness of males to different female body parts. In the first, the head and mesosoma (thorax plus first abdominal segment) were tested against the metasoma (remaining abdominal segments), and in the second the genital capsule (terminal abdominal segment plus ovipositor) was tested against the remaining anterior portion of the metasoma.

A glass microscope slide was divided into two halves and the two body parts being tested were placed randomly in the middle of either half, presenting a choice to the males. The slide was clamped at its midpoint and held horizontally at a

height of 30 cm by a small metal stand. Tests were conducted in a flight tunnel (Keller 1990) at a wind speed of 30 cm/s. Males were kept in a separate cage and removed for testing individually with a minimum of disturbance. They were released 30 cm downwind of the slide and observed to determine (1) which half of the slide they landed on, (2) whether subsequently they oriented to either body segment and performed typical elements of courtship (Field and Keller 1993), and (3) whether they attempted to copulate with either body segment.

The same males were then tested again, but this time the female body segments were washed in diethyl ether prior to testing. This solvent removed all contaminating pheromone from the surface of the body segment (Golub and Weatherston 1984). Therefore any subsequent emission of pheromone could be due only to continued leaking of the pheromone onto the surface from an internal glandular reservoir. The preference of courting males for a particular segment after washing would therefore localize the pheromone gland to somewhere within that segment.

Most males were successful in navigating to the slide; those that did not were discarded after a few attempts. To lessen the effects of pheromone contamination and depletion of pheromone reserves, the stand was washed with diethyl ether and body segments and slides were replaced at regular intervals throughout the course of the experiments. Variation in attractiveness of females was assumed to be negligible. Fifty males were tested in each experiment and data were analysed using two-tailed binomial tests (Zar 1984).

Scanning Electron Microscopy (SEM).—Two methods were used to prepare specimens for SEM observations. In the first, newly-dissected specimens were immersed in Peterson's KAA (kerosene (10%), 95% alcohol (75%), glacial acetic acid (15%)) prior to washing in 0.1 M phosphate buffer and dehydration through an alcohol series. This technique is commonly used to preserve larval insects because it distends structures that may otherwise lose shape (Smithers 1981).

The second method was to "clear" the specimens by gently warming in 10% KOH for 30-60 minutes. This removed most tissue and allowed dissection of the specimens so that both internal and external cuticular structures could be examined under SEM. Following dehydration, the KAA-treated and cleared specimens were critical-point dried in an EMSCOPE CPD 750 and mounted on TAAB aluminium stubs using Acheson Electrodag 915. They were coated by vacuum evaporation with carbon and gold palladium and examined in an ETEC Autoscan scanning electron microscope at an accelerating voltage of 20 kV.

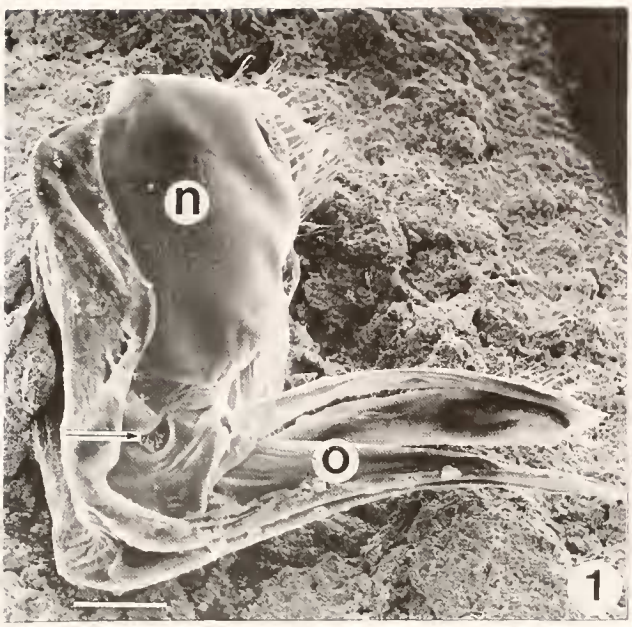
Light Microscopy (LM).—Genital capsules were removed and immersed for 4 hours in fixative (3% glutaraldehyde/3% formaldehyde made up in 0.1 M phosphate buffer, pH 7.4, to which had been added 2.5% polyvinyl pyrrolidone). They were washed in 0.1 M phosphate buffer overnight and then dehydrated by passing through a series of alcohols. After washing in propylene oxide, they were infiltrated with increasing concentrations of TAAB epoxy embedding resin over 48 hours and then embedded in resin by curing at 60° C for a further 48 hours.

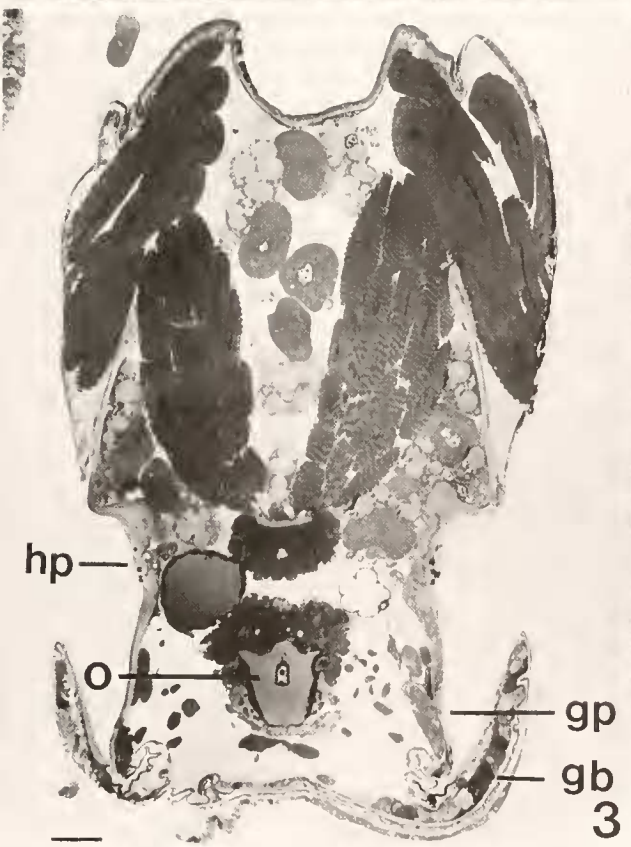
Using glass knives in a Sorvall MT2-B "Porter-Blum" ultramicrotome, serial transverse sections of 0.5 µm thickness were cut, starting from near the base of the ovipositor and proceeding anteriorly. These were stained using 0.025% Toluidine Blue in 0.5% Borate buffer and photographed with a Wild MPS 45 photoautomat on Ilford Pan-f 50 ASA film. Agfa x 3 light green and Wratten 58 filters were used to achieve suitable contrast. Males were also subject to the same processing procedures and serial sections were cut from the posterior tip of the abdomen.

Transmission Electron Microscopy (TEM).-Tissue samples were prepared in the same manner as for LM. Gold to silver sections (0.08 mm) were cut with a Diatome diamond knife using a Reichert-Jung Ultracut and stained with saturated uranyl acetate in 70% alcohol for 20 minutes, followed by lead citrate for 12 minutes (Reynolds, 1963). They were examined in a JEOL 100S transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS

Behavioural Experiments.—In the first experiment, neither the head/mesosoma nor the metasoma was found to be more attractive when the segments had not been washed in ether. However, when surface contamination had been removed with ether, males responded preferentially toward the metasoma, indicating that it was the source of the sex pheromone (Table 1).





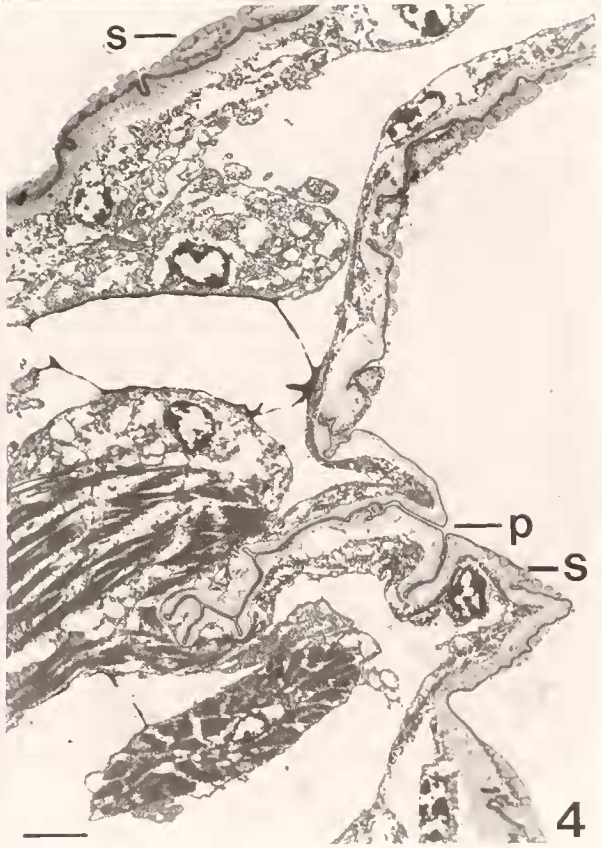


Fig. 1. SEM of lateral view of genital capsule in *Cotesia rubecula*. Arrow indicates position of hair plate and pores (see Fig. 2); n - ninth tergite; o - ovipositor. Scale bar = 100 µm

Fig. 2. High magnification SEM of hair plate and pores. Scale bar = 10 µm

Fig. 3. Transverse section of genital capsule, showing under LM two possible sex pheromone-producing glands: gp - glands associated with surface pores; gb - gland at base of genital capsule; hp - hair plate. Scale bar = 30 µm

Fig. 4. TEM of basal section of genital capsule. p - pore canals; s - secretory droplets. Scale bar = 5 µm

Table 1. Response of male *Cotesia rubecula* to a choice between the metasoma and the head+mesosoma of females, washed in ether or unwashed. * indicates significant differences (P<0.001).

		Number Responding	
Treatment	Response	Metasoma	Head+Mesosoma
Unwashed	Land	23	27
	Court	23	27
	Copulate	10	22
Washed	Land	36	1*
	Court	36	1*
	Copulate	19	1*

Table 2. Response of male *Cotesia rubecula* to a choice between the genital capsule and the anterior portion of the metasoma of females, washed in ether or unwashed. * indicates significant differences (P<0.001).

		Number Responding	
Treatment	Response	Anterior Metasoma	Genital Capsule
Unwashed	Land	17	31
	Court	18	27
	Copulate	19	1*
Washed	Land	3	28*
	Court	4	21*
	Copulate	3	1

In the second experiment, there was no preference shown by males for either the unwashed genital capsule or the unwashed anterior portion of the metasoma in terms of frequencies of landing and courting (Table 2). Several males moved between the body segments after landing in close proximity to one of them. More males attempted to copulate after courting an unwashed metasoma (18/18, plus one that courted to the genital capsule initially) than an unwashed genital capsule (1/27). After these body parts were washed with ether, males showed a clear preference for the genital capsule in terms of landing and courting, but not copulating.

Microscopy.—Having identified the genital capsule as the source of the sex pheromone, we examined KAA-treated genital capsules under the SEM to determine whether there were any surface pores that could serve as outlets for pheromone secretion. The only pores found were positioned slightly below and posterior to a mechanosensory hair plate (Figs. 1, 2). Dissection of a cleared specimen revealed the internal attachment site of these mechanosensory hairs to be at the base of the second valvifer, which is an internal cuticular plate comprising part of the ovipositor system (see Snodgrass, 1935).

The LM sections revealed only one region of tissue that was a possible candidate for the sex pheromone source. A pair of glands was located in a position corresponding closely to that of the pores, i.e., just below the hairs and appearing immediately before the hairs in serial transverse sections (Fig 3). No pores were apparent, but this

may have been a result of the small proportion of sections cut (approx. 1/10) that were actually retrieved, stained and mounted for examination. The results of ultrastructural examination of this gland were inconclusive and failed to offer any indication of the organelles present or whether it was secretory in nature.

TEM examination revealed the presence of a second pair of secretory glands (Fig. 4), located at the base of the genital capsule and adjacent to folds of membrane which are presumably stretched and compressed during movements of the ovipositor. The ultrastructure of these glands was also indistinct, but pore canals leading to the surface and an abundance of secretory bodies were visible on or just under the surface of the cuticle.

DISCUSSION

The behavioural experiments clearly demonstrate that the genital capsule is the source of the sex pheromone in *C. rubecula*. With all surface pheromone removed, males were attracted to the metasoma in preference to the head/mesosoma and, when given a further choice, made their initial approach more often to the genital capsule rather than to the anterior portion of the metasoma. This is indicative of the continuous emission of pheromone from an internal reservoir located in the genital capsule, which provides an olfactory cue to the searching wasp.

There are, however, two anomalous results which require explanation. Firstly, a single male chose the washed head/mesosoma over the metasoma in the first experiment. This could have been due to incomplete washing of the body parts. Secondly, although significantly more males landed near and courted the genital capsule as opposed to the anterior portion of the metasoma, they attempted copulation with the genital capsule less frequently. This is not considered to be evidence that the sex pheromone gland is in the anterior portion of the metasoma. Rather, it suggests that a visual cue is involved in triggering attempts at copulation by the male. Upwind flight, landing, orienting and courting all appear to be stimulated entirely by chemical cues. The presence of a particular threshold amount of chemical may "prime" males so that they are ready to mount and copulate after a certain amount of courtship, but an object of appropriate size, shape and colour in the vicinity of a pheromone source may be required to release copulation behavior. One male did attempt copulation with the tiny genital capsule (0.5 mm in length), so the pheromone alone may be sufficient to elicit attempts at copulation in some cases.

The histology and electron microscopy failed to resolve the exact location of the sex pheromone gland within the genital capsule, but narrowed the possibilities to two structures. One of these is a group of cells located directly underneath surface pores which are adjacent to the mechanosensory hair plate on the genital capsule (Figs. 1-3). The hair plate was in turn juxtaposed to the second valvifer, which places this gland in approximately the same position as the putative pheromone glands indicated by Tagawa (1977, 1983) for a number of other closely-related braconids. Although this constitutes circumstantial evidence that this is the sex pheromone gland in *C. rubecula*, the absence of any clearly defined secretory products in the ultrastructural examinations leaves some uncertainty. The pores associated with these glands required thorough washing before they became visible by SEM.

Another possible source of sex pheromone is a gland located further toward the base of the genital capsule. The most striking feature of this gland is an abundance of associated secretory bodies amassed on and under the surface of the cuticle which are visible under TEM (Fig. 4). One possibility is that these secretions act as a lubricant for the ovipositor as it is extended and retracted from the metasoma. However, if it is the sex pheromone gland, then such movements would undoubtedly smear the secretions over the cuticle, which would be ideal for pheromone dispersal. An additional possibility is that the one secretion has the dual function of lubrication and sex attractant. In the absence of isolation and identification of the pheromone, the question of which of the two glands is the source of sex pheromone in *C. rubecula* will remain unresolved.

ACKNOWLEDGEMENTS

We thank Chris Leigh for the generous use of his facilities for histological processing, preparation of TEM sections and help with the SEM, Dr Adam Lockett for the use of his photographic equipment, Frances FitzGibbon for printing the micrographs and Paul Dangerfield for proofreading the manuscript.

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